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Mapping Oxidative DNA Damage at Nucleotide Level

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DNA damage induced by reactive oxygen species (ROS) is considered an important intermediate in the pathogenesis of human conditions such as cancer and aging. By developing an oxidative-induced DNA damage mapping version of the Ligation-mediated polymerase chain reaction (LMPCR) technique, we investigated the *in vivo* and *in vitro* frequencies of DNA base modifications caused by ROS in the human *p53* and *PGK1* gene. Intact human male fibroblasts were exposed to 50 mM H_2O_2, or purified genomic DNA was treated with 5 mM H_2O_2, 100 μM Ascorbate, and 50 μM, 100 μM, or 100 μM of Cu(II), Fe(III), or Cr(VI) respectively. The damage pattern generated *in vivo* was nearly identical to the *in vitro* Cu(II) or Fe(III) damage patterns; damage was non-random with guanine bases heavily damaged. Cr(VI) generated an *in vitro* damage pattern similar to the other metal ions, although several unique thymine positions were damaged. Also, extra nuclear sites are a major contributor of metal ions (or metal-like ligands). These data show that the local probability of H_2O_2-mediated DNA damage is determined by the primary DNA sequence, with chromatin structure having a limited effect. The data suggest a model in which DNA–metal ion binding domains can accommodate different metal-ions. LMPCR's unique aspect is a blunt-end ligation of an asymmetric double-stranded linker, permitting exponential PCR amplification. An important factor limiting the sensitivity of LMPCR is the representation of target gene DNA relative to non-targeted genes; therefore, we recently developed a method to eliminate excess non-targeted genomic DNA. Restriction enzyme-digested genomic DNA is size fractionated by Continuous Elution Electrophoresis (CEE), capturing the target sequence of interest. The amount of target DNA in the starting material for LMPCR is enriched, resulting in a stronger amplification signal. CEE provided a 24-fold increase in the signal strength attributable to strand breaks plus modified bases created by ROS in the human *p53* and *PGK1* genes, detected by LMPCR. We are currently taking advantage of the enhanced sensitivity of target gene-enriched LMPCR to map DNA damage induced in human breast epithelial cells exposed to non-cytotoxic concentrations of H_2O_2.

Keywords: DNA damage, hydrogen peroxide, oxyradicals, cancer, aging

Abbreviations: μg, microgram, μl, microliter, *PGK1*, Phospho-Glycerate Kinase, kb, kilobase, CEE, continuous elution electrophoresis, LMPCR, ligation-mediated polymerase chain reaction, ROS, reactive oxygen species, H_2O_2, hydrogen peroxide, Fpg, FormamidoPyrimidine DNA glycosylase, Nth, Endonuclease III

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INTRODUCTION

DNA damage induced by reactive oxidation species (ROS) is an important intermediate in the pathogenesis of human conditions such as cancer and aging.[1,2] ROS-induced DNA damage products are both mutagenic and cytotoxic.[3] One commonly studied ROS is that produced by H_2O_2 in the presence of transition metal ions. The mutational spectra of H_2O_2[4,5] and the transition metal ions Fe, Cu,[6,7] and Cr[8] have been studied in model systems, but the relationship of induced DNA damage to these spectra remains unknown. Knowledge of this relationship is crucial in order to extrapolate from model mutational analysis systems to endogenous mutational spectra generated *in vivo*. This is of significant scientific and medical interest since elucidation of the role oxidatively-induced DNA damage has in carcinogenesis could ultimately enable one to develop rational therapeutic interventions beneficial in the prevention of human malignancies.

Until recently, progress in this area has been hampered by the lack of damage measurement techniques with nucleotide resolution. Ligation-mediated polymerage chain reaction (LMPCR) is a genomic sequencing method for mapping of rare DNA single-stranded breaks. In a previous report, we produced a map of DNA base damage caused by Cu(II)/ascorbate/H_2O_2 in purified DNA *in vitro* at nucleotide resolution.[9] We showed that the DNA base damage pattern was distributed non-uniformly in the promoter region of the human *PGK1* gene, with certain sequence motifs being highly susceptible to damage. Certain questions remain. How does the *in vitro* Cu(II)/ascorbate/H_2O_2 base damage pattern compare to the base damage pattern induced *in vivo* by exposure of intact target cells to H_2O_2? Can metal ions other than copper produce a similar mutational pattern? Furthermore, if other metal ions or metal-like ligands contribute to the *in vivo* damage pattern, do they normally reside in proximity to DNA or are they recruited from extranuclear sites? These questions are addressed in this report.

Also addressed are recent improvements to the LMPCR procedure. Prior to performing LMPCR, the amount of target DNA in the starting material is enriched, resulting in a stronger amplification signal. This and current enzyme improvements to the LMPCR protocol are discussed.

MATERIALS AND METHODS

In Vivo H_2O_2 Treatment of Human Skin Fibroblasts

Human male foreskin fibroblasts were grown in 150 mm dishes to confluent monolayers in Dulbecco's Modified Eagle Medium containing 10% (v:v) fetal bovine serum. Fibroblasts were treated with serum-free Minimum Essential Medium with 1 mM sodium phosphate containing 50 mM H_2O_2 at 37°C for 30 min. After washing, cells were harvested and DNA was isolated as previously described.[10]

Human male skin fibroblast DNA was prepared for *in vitro* assay as previously described.[9] After phenol/chloroform extraction, the DNA was precipitated in ethanol, redissolved in 10 mM HEPES, 1 mM EDTA, pH 7.4 at 70 μg/ml, then dialyzed against distilled water overnight at 4°C.

Metal Ion–Ascorbate–H_2O_2 Treatment

Ten μg of dialyzed DNA dissolved in 161 ml of H_2O was incubated at room temperature for 30 min with 50 μM $CuCl_2$, 100 μM $FeCl_3$, or 100 μM $K_2Cr_2O_7$. Chelex® treated potassium phosphate, pH 7.5 (± 0.3 M sucrose), ascorbate, and H_2O_2 were added to final concentrations of 1 mM, 100 mM, and 5 mM, respectively. After 30 min at room temperature with gentle rocking, the reaction was quenched by the addition of EDTA to 2 mM, followed by precipitation of DNA in 0.3 M sodium acetate, pH 7.0 and 2 volumes of cold ethanol.

Isolation and Treatment of Fibroblast Nuclei

Human male foreskin fibroblasts were grown in 150 mm dishes to confluent monolayers in Dulbecco's Modified Eagle Medium containing 10% (v:v) fetal bovine serum. After removal of medium and washing with 25 ml of a 154 mM NaCl solution, cells were lysed by the addition of 10 ml buffer A (0.3 M sucrose, 60 mM KCl, 15 mM NaCl, 60 mM Tris-HCl, pH 7.4, 2 mM EDTA) containing 0.5% Nonidet-P40 (has now replaced with IGEPAL-CA-620, ICN Biochemicals, Aurora, Ohio USA). Nuclei were collected by centrifugation at $1000 \times g$ for 10 min at room temperature, then gently resuspended in sucrose/phosphate buffer (1 mM potassium phosphate, pH 7.5, 60 mM KCl, 15 mM NaCl, 0.3 M sucrose). After two additional washes in sucrose/phosphate buffer, nuclei were exposed to 50 mM $H_2O_2 \pm 50\,\mu M$ Cu(II) or $50\,\mu M$ Fe(III) $\pm 100\,\mu M$ ascorbate in sucrose/phosphate buffer for 30 min at 37°C. Reactions were quenched by the addition of EDTA or desferrioximine (for iron-containing samples) to 2 mM. Treated nuclei were then collected by centrifugation, resuspended in 4 ml buffer A and DNA was isolated as described previously.[10]

Ligation-mediated Polymerase Chain Reaction

Digestion of treated DNA with Nth and Fpg proteins[9] and fragment size analysis by glyoxal gel electrophoresis[10] have been described in detail elsewhere. The LMPCR technique (Figure 1) has been described in detail elsewhere.[9] Key steps with recent enzyme modifications such as the incorporation of a hot-start by using AmpliTaq® Gold polymerase to provide better signal-to-noise ratio and cycling modifications are as follows:

Primer Extension

Primer 1 is extended in a siliconized 0.65 ml tube: a thermocycler (MJ Research Inc., Watertown, MA, USA) is used for all incubations. DNA (0.5–1.3 µg) is diluted in a volume of 15 µl of a solution containing 40 mM Tris-HCl, pH 7.7, 50 mM NaCl and 0.75 pmol of Primer 1. DNA is denatured at 98°C for 3 min and the primer (Primer 1) annealed at 45°C for 30 min (primers for the *PGK1* housekeeping gene are in,[9] and *p53* gene in.[11] After cooling the sample to 4°C, 9 µl of the following mix is added: 7.5 µl of $MgCl_2$–dNTP mix (20 mM $MgCl_2$, 20 mM dithiothreitol and 0.25 mM of each dNTP), 1.1 µl dH_2O and 0.4 µl of Sequenase® 2.0 (13 U/µl, U.S. Biochemicals, Cleveland, Ohio USA). Samples are then incubated at 48°C for 15 min. The samples are placed on ice and 6 µl ice-cold 310 mM Tris-HCl, pH 7.7, is added. To inactivate Sequenase® samples are incubated at 67°C for 15 min. Samples are placed on ice.

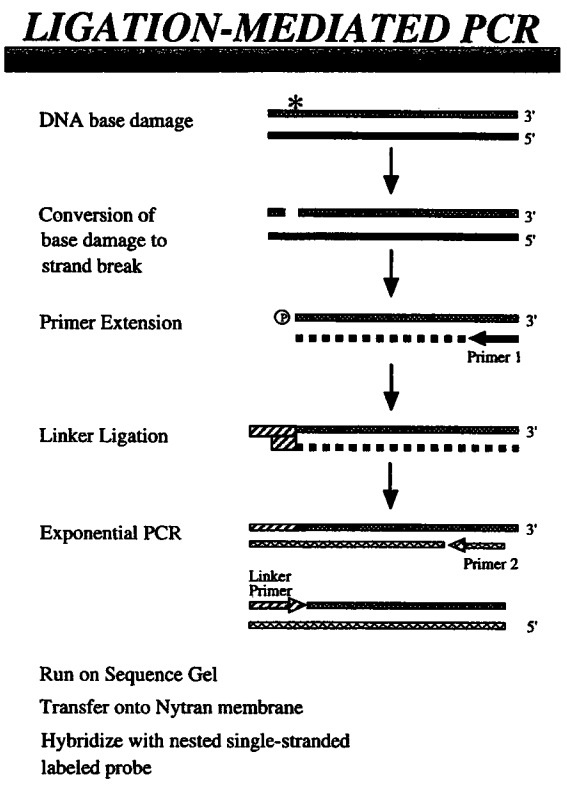

FIGURE 1 Schematic representation of the steps in DNA base damage mapping by LMPCR. Reproduced with permission.

Ligation

The primer-extended molecules that have a 5′ phosphate are ligated to an unphosphorylated asymmetric double-stranded linker.[12] To each sample (consisting of 30 µl), 45 µl of the following ligation mix is added: 13.33 mM $MgCl_2$, 30 mM dithiothreitol, 1.7 mM ATP, 83.3 µg/ml BSA, 100 pmol of linker and 5 U of T_4 DNA ligase (5 U/µl, Boehringer Mannheim, Gaithersburg, MD, USA). Samples are incubated overnight at 18°C. Ligase is inactivated by incubation at 70°C for 10 min. Samples are placed on ice. Next, 25 µl of 10 M ammonium acetate, 1 µl of 0.5 M EDTA, pH 8.0, 1 µl of 20 µg/µl glycogen, followed by 250 µl of ice-cold ethanol to precipitate the DNA. DNA pellets are redissolved in 50 µl of dH_2O.

PCR Amplification

Fifty µl of an AmpliTaq® Gold polymerase mix (2 × AmpliTaq® Gold reaction buffer (Perkin Elmer Inc., Foster City, CA, USA), 1 mM $MgCl_2$, 400 µM of each dNTP, 10 pmol of primer 2, 10 pmol of linker primer[12] and 3.0 U of AmpliTaq Gold polymerase (5 U/µl, Perkin Elmer Inc., Foster City, CA, USA) is added to each sample, and reactions are overlaid with mineral oil. (Note: 2 × AmpliTaq® Gold buffer contains 3 mM $MgCl_2$. Therefore, the final $MgCl_2$ concentration in the 50 µl 2 × AmpliTaq® Gold polymerase mix is 4 mM, due to the added 1 mM $MgCl_2$. This translates into a 2 mM final $MgCl_2$ concentration in the 100 µl PCR reaction). Reactions undergo 1 PCR cycle of 95°C for 8 min (activating AmpliTaq® Gold polymerase), 95°C for 4 min, T_m of Primer 2 for 2 min, and 72°C for 3 min, 1 PCR cycle of 95°C for 4 min, T_m of Primer 2 for 2 min, and 72°C for 3 min, 18 PCR cycles of 95°C for 1 min, 1°C below T_m of Primer 2 for 2 min, and 72°C for 3 min. Lastly, an extension is performed at 72°C for 10 min. Following the PCR reaction, a stop mix (13 µl of 3 M sodium acetate, pH 5.2, 3 µl of 0.5 M EDTA, pH 8.0, and 9 µl of dH_2O) is added under the mineral oil layer. Samples are extracted with 170 µl of premixed phenol : chloroform (50 : 120 µl), then ethanol precipitated by adding 370 µl ice-cold ethanol. Air-dried DNA pellets are dissolved in 7.0 µl of premixed formamide-dye (2.3 µl dH_2O, 4.7 µl formamide loading dye: 95% (v/v) formamide, 10 mM EDTA, pH 8, 0.05% (w/v) xylene cyanol, 0.05% (w/v) bromophenol blue) in preparation for sequencing gel electrophoresis.

Continuous Elution Electrophoresis

The CEE procedure has been described in detail.[13] Several key steps are:

BamH I Digestion of Total Genomic DNA

Following ROS treatment, 50 µg aliquots of DNA were digested in 250 U *Bam*H I (Gibco BRL, Gaithersburg, MD, USA) and 10 µg RNase A (Sigma, St. Louis, MO, USA), in a volume of 500 µl at 37°C for 4 h. Digestion was carried out in Gibco BRL 1× React 3 enzyme buffer. Digestions were stopped by the addition of 10 µl 0.5 M EDTA. Phenol (AquaPhenol; Appligene/ONCOR, Gaithersburg, MD, USA)/phenol–chloroform/chloroform (J.T. Baker, Phillipsburg, NJ, USA) extractions were carried out to remove the proteins. DNA was precipitated by the addition of 50 µl of 3 M NaOAc, pH 7, and 1 ml of ice-cold ethanol. Air-dried pellets were resuspended in 50 µl 1× TE (10 mM Tris-HCl, pH 8, 1 mM EDTA), and prepared for continuous elution electrophoresis.

Loading the DNA Sample and Running the Continuous Elution Electrophoresis Apparatus

Thirty µl of 10× agarose loading dye (Sigma, St. Louis, MO, USA) were added to 300 µg of *Bam*H I-digested (Gibco BRL, Gaithersburg, MD, USA) genomic DNA in a volume of 300 µl TE (final concentration of 1 µg/ml). The DNA sample was loaded onto a Model 491 Prep Cell (Bio-Rad Laboratories, Hercules, CA, USA), containing a 0.5% preparative agarose gel (SeaKem®

Gold; FMC BioProducts, Rockland, ME, USA) and a 0.25% agarose stacking gel (SeaKem® Gold; FMC BioProducts, Rockland, ME, USA). The sample was run in 50 mM TBE (Tris-borate/EDTA), at 55 constant volts (PowerPac 300 Power Supply; Bio-Rad Laboratories, Hercules, CA, USA) at 4°C. An elution flow rate of 50 µl per minute was maintained by a peristaltic pump (Model EP-1 Econo Pump; Bio-Rad Laboratories, Hercules, CA, USA), with fraction collection times of 20 min (1 ml final volume).

Fraction Viewing by Standard Agarose Electrophoresis (Optional)

After continuous elution electrophoresis, 30 µl from each fraction (representative of 90 ng of DNA in 50 mM TBE buffer) were mixed with 3 µl of 10 × agarose loading dye (Sigma, St. Louis, MO, USA) and loaded onto 0.7% standard agarose gels (SeaKem® LE, FMC BioProducts, Rockland, ME, USA) and run in 50 mM TBE, at 100 constant volts. Gels were stained by containing ethidium bromide (0.5 µg/ml), then photographed using Polaroid 667 film (Polaroid Corporation, Cambridge, MA, USA). DNA concentration was determined by A_{260} measurements.

Fraction Screening for Gene of Interest by Dot-Blot Analysis

After continuous elution electrophoresis, 10 µl from each fraction (representative of 30 ng of DNA in 50 mM TBE buffer) were added to 190 µl of 0.4 M NaOH, 10 mM EDTA solution. Samples were heated to 95°C for 5 min in a thermocycler (PTC-100, MJ Research, Watertown, MA, USA), placed on ice, and loaded onto a Dot-Blot apparatus (Bio-Dot; Bio-Rad Laboratories, Hercules, CA, USA) containing a positively charged nylon plus membrane (Qiabrane; Qiagen, Santa Clarita, CA, USA). Wells were rinsed with 200 µl of 0.4 M NaOH, 10 mM EDTA solution. Membrane was then soaked in 2 × SSC (NaCl/sodium citrate) for 5 min, UV-cross linked (1200 J/m^2) (Stratalinker; Stratagene, La Jolla, CA, USA), and placed into a hybridization tube containing hybridization solution (0.25 M $NaPO_4$, 1 mM EDTA, 7% SDS (sodium dodecyl sulfate), 1% BSA (Bovine Serum Albumin) (fraction V; Sigma, St. Louis, MO, USA)), and radiolabeled probe.[13] This was placed into a hybridization oven (HB 1100D Red Roller II, Pharmacia Biotech, Piscataway, NJ, USA) overnight.[9] After overnight hybridization at 66°C, membranes were washed for 5 min in Buffer A (20 mM $NaPO_4$, 1 mM EDTA, 2.5% SDS, 0.25% BSA (fraction V; Sigma, St. Louis, MO, USA)) at 66°C, followed by 5 min in Buffer B (20 mM $NaPO_4$, 1 mM EDTA, 1% SDS) at 66°C. Buffer B wash was repeated two times. Air-dried membranes were exposed to Kodak XAR-5 X-ray films (Eastman Kodak, Rochester, NY, USA) with intensifying screens (Optex, Cedar Knolls, NJ, USA) at −70°C. The intensity of each dot was quantitated by PhosphorImager analysis (Model 425S; Molecular Dynamics, Sunnyvale, CA, USA).

Cleavage of Enriched Samples at ROS-induced Modified Bases

Following ROS treatment, continuous elution electrophoresis and fraction screening by dot-blot analysis, fractions containing the highest percentage of the gene of interest were precipitated by making two tubes, each consisting of 500 µl eluted fraction, 50 µl 3 M NaOAc, pH 7, 1 µl glycogen (20 µg/µl), and 1 ml ice-cold ethanol. DNA was ethanol precipitated by a 10 mm incubaion on dry ice. Air-dried pellets were resuspended in 25 µl 1× TE, pH 8. Each respective pair was pooled to yield a 50 µl total volume consisting of 3 µg DNA. Respective fractions (containing 3 µg DNA) were then mixed with 50 µl of a unique 2× Nth/Fpg reaction buffer (91.4 mM Tris-HCl, pH 7.7, 200 mM KCl, 1.1 mM EDTA, 0.2 mM DTT, 200 µg/µl BSA-fraction *V*), yielding a final volume of 100 µl. To this, 400 ng Fpg protein (FormamidoPyrimidine DNA Glycosylase) from *Escherichia coli* and

100 ng Nth protein (Endonuclease III) from *Escherichia coli* was added and samples were digested at 37°C for 60 min as previously described.[9] Fpg and Nth proteins were provided by Dr. Timothy R. O'Connor of the Beckman Research Institute of the City of Hope (Duarte, CA, USA). Control samples (no enzyme) were incubated in buffer alone (data not shown). Digestions were terminated as previously described.[9] Finally, the DNA pellets were dissolved in Sequenase buffer (40 mM Tris-HCl, pH 7.7 and 50 mM NaCl) for LMPCR, as previously described.[9]

RESULTS

Distribution of DNA Damage Induced *In Vitro* by Cu(II), Fe(III), or Cr(VI) Plus H_2O_2/ascorbate and *In Vivo* by H_2O_2

The distribution of oxidative damage induced in exons 5 and 9 of human *p53* and the promoter region of human *PGK1* was assessed by LMPCR.[14] The autoradiograms indicating the damage distributions induced in the region of the human *PGK1* gene covered by primer set A[9] and in exon 9 of the human *p53* gene[11] are shown in Figure 2A and B, respectively. In these regions of the genome, the base damage

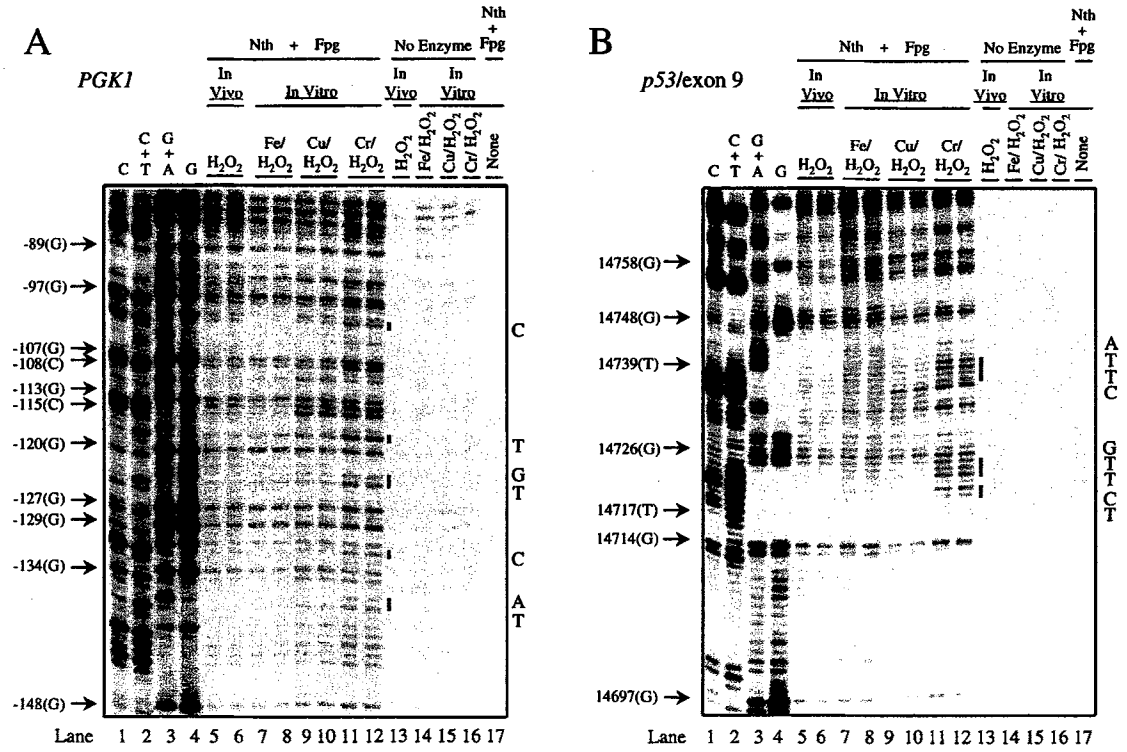

FIGURE 2 LMPCR analysis of damage induced in (A): The promoter region of human *PGK1* using primer set A (transcribed strand) and (B): Exon 9 of human *p53* (transcribed strand). Lanes 1–4, DNA treated with standard Maxam-Gilbert cleavage reactions. Lanes 5, 6, 13, DNA recovered from intact human foreskin fibroblasts exposed to 50 mM H_2O_2. Lanes 7–8, 14, dialyzed genomic DNA treated with 100 μM Fe(III)/100 μM ascorbate/5 mM H_2O_2 in the presence of 0.3 M sucrose. Lanes 9, 10, 15, DNA treated with 50 μM Cu(II)/100 μM ascorbate/5 mM H_2O_2 in the presence of 1 mM potassium phosphate buffer. Lanes 11, 12, 16, DNA treated with 50 μM Cr(VI)/100 μM ascorbate/5 mM H_2O_2. Lane 17, DNA incubated in potassium phosphate buffer and digested with Nth and Fpg proteins. The DNA in lanes 5–12 was digested with Nth and Fpg proteins after treatment; the DNA in lanes 13–16 was incubated in digestion buffer alone after treatment. Positions of high damage frequency bases are marked with arrows to the left of lane 1. The sequence of positions heavily damaged in the presence of chromium, but not copper or iron, is denoted by rectangles to the right of lane 12. Reproduced with permission.

frequency distributions induced in dialyzed DNA *in vitro* by Cu(II) and Fe(III) plus ascorbate/H_2O_2 are nearly identical. Sucrose was included in the Fe(III) reaction to suppress the direct strand break signal. Sucrose has no effect on the LMPCR-derived damage distribution signals.[14] The base damage distribution associated with these two transition metal ions is non-uniform, confirming our previous observations[9] with Cu(II). Prominent base damage hotspots are observed in *PGK1* and in *p53* exon 9 (Figure 2A and B).

Tabulations of the damage frequencies induced in exons 5 and 9 of *p53* and the other region of the *PGK1* promoter were covered.[14] The distribution of DNA base damage occurring in both of these regions by *in vitro* Cu(II)- and Fe(III)-mediated damage, were nearly identical. The distribution of damage caused by Cr(VI)/ascorbate/H_2O_2 was similar, but not identical, to that mediated by copper or iron ions in these regions. The unique chromate sensitive positions were often thymines (Figure 2A and B).

The distribution of DNA base damage occurring *in vivo* induced by exposure of cultured human male fibroblasts to 50 mM H_2O_2 was also determined in *PGK1* and *p53*.[14] Here, 50 mM H_2O_2 induces a global damage frequency in human male fibroblast DNA *in vivo* equivalent to the damage frequency induced *in vitro* by 50 μM Cu(II)/100 μM ascorbate/5 mM H_2O_2 (data not shown). Figure 2A and B demonstrate that the base damage distribution induced *in vivo* in the assessed regions of *PGK1* and *p53* were identical to the damage distributions induced *in vitro* by Cu(II) or Fe(III) plus H_2O_2/ascorbate and similar to the damage distribution induced *in vitro* by Cr(VI)/H_2O_2/ascorbate. Control experiments, such as extending the number of cell rinses with Chelex®-treated phosphate-buffered saline after H_2O_2 exposure to as many as 9 times, resulted in no alteration of the damage frequency. This demonstrates that the putative *in vivo* damage distribution was not a post-DNA extraction artifact. Furthermore, the concentration of H_2O_2 in the medium at the end of the 30 min incubation period was well below that required to observe damage signals by LMPCR.

Comparison of damage intensity among sequence contexts were made by applying sequential Wilcoxon rank-sum tests.[14] Guanine is the most easily modified base associated with H_2O_2-mediated DNA damaging reactions both *in vivo* and *in vitro*. The triplet d(pCGC) is the principal hotspot sequence.

Damage Induced by H_2O_2 in Isolated Fibroblast Nuclei

Exposure of human male fibroblasts to a concentration of H_2O_2 several orders of magnitude higher than those generated under basal metabolic conditions was required in order to generate sufficient DNA base damage for the purpose of damage frequency mapping by LMPCR. Therefore, we assessed to what extent artifacts caused by exposure to high concentration H_2O_2 contributed non-physiologic distortion of the observed damage frequency patterns. The principal effect of high concentration H_2O_2, causing severe oxidative stress, turned out to be release of cellular transition metal ions from normally sequestered extranuclear sites.

Evidence for transition metal ion release from extranuclear sites by the severe oxidative stress was obtained by assessing DNA damage in isolated human male fibroblast nuclei. DNA damage was assessed globally by neutral denaturing agarose gel electrophoresis.[10] Figure 3 shows that isolated nuclei behave similar to naked DNA in that neither H_2O_2 alone nor Cu(II)/Fe(III) + ascorbate cause detectable DNA damage in isolated human male fibroblast nuclei. However, nuclei behave differently from naked DNA in that significant DNA damage was observed if H_2O_2 and Cu(II)/Fe(III) were added. In fact, base damage induced by H_2O_2/copper ion in the isolated nuclei was greater than that induced *in vivo* by equimolar H_2O_2 with or without supplemental 50 μM Cu(II) in

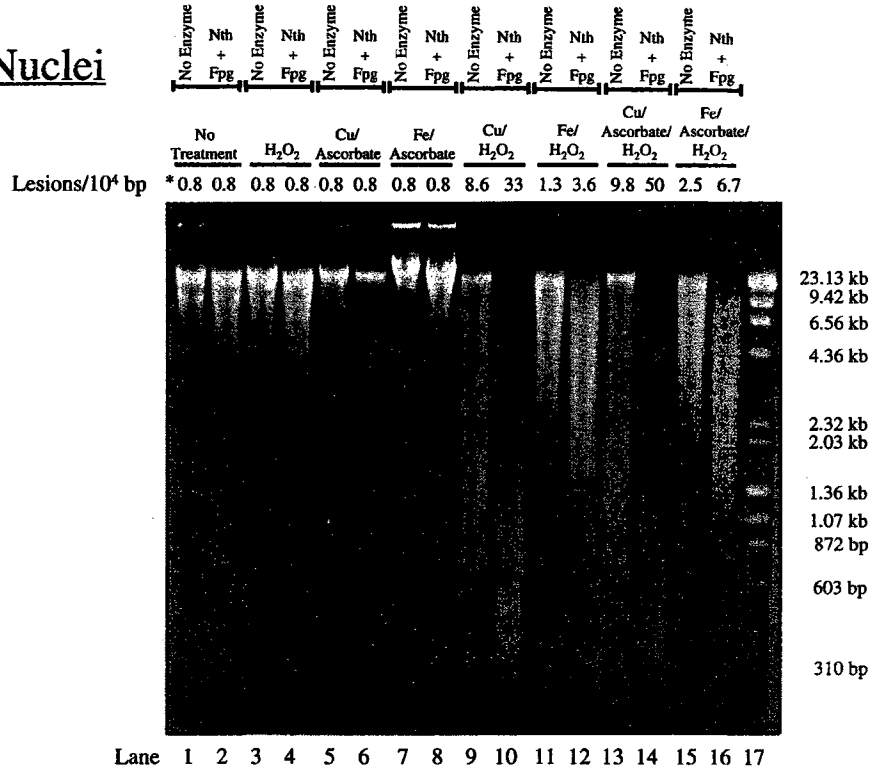

FIGURE 3 Global frequency of direct strand breaks ("No Enzyme" lanes) and direct strand breaks plus modified bases ("Nth + Fpg" lanes) observed after exposure of isolated human male fibroblast nuclei to 50 mM H_2O_2 (lanes 3, 4), 50 μM Cu(II) + 100 μM ascorbate (lanes 5, 6), 50 μM Fe(III) + 100 μM ascorbate (lanes 7, 8), or combinations of these reagents (lanes 9–16). Lane 17, 500 ng lambda DNA digested with *Hind* III and 500 ng PhiX174 digested with *Hae* III. Lanes 1, 2, nuclei not treated (Controls). Nuclear isolation was performed as described in Ref. [14] Exposures were for 30 min at 37°C, after which DNA was isolated. Electrophoresis was carried out as described in Ref. [10]. (*) 0.8 is a lower limit of estimated lesion frequency in these lanes; a more precise value could not be determined. Reproduced with permission.

the medium.[9] Thus, isolated nuclei have the following two notable properties: (1) they contain endogenous reducing agents capable of reducing transition metals such that the metals redox cycle in the presence of H_2O_2, and (2) they do not contain sufficient bound metals (or metal-like ligands) to cause significant base damage in the presence of H_2O_2.

Enhancement of LMPCR Damage Detection Sensitivity by Genomic Gene Enrichment

The requirement for exposure of fibroblasts to cytotoxic concentrations of H_2O_2 in order to map ROS-induced DNA base damage *in vivo* by LMPCR spurred us to develop methods to enhance the sensitivity of LMPCR. One approach to enhancing LMPCR-generated base damage signal intensity is to increase the relative copy number of the target gene in the substrate genomic DNA. This was accomplished by size-fractionating restriction endonuclease-digested, ROS-exposed genomic DNA by continuous elution electrophoresis (CEE) through a preparative agarose gel (Figure 4, Ref. [13]) as follows: Total human genomic DNA was damaged by exposure to 0.5 mM H_2O_2 in the presence of 50 μM Cu(II) and 100 μM ascorbate, then digested with *Bam*H I. Three hundred microgram aliquots were fractionated by CEE. One ml fractions were collected, viewed on agarose gels – an optional step (Figure 5A and B), and fractions containing

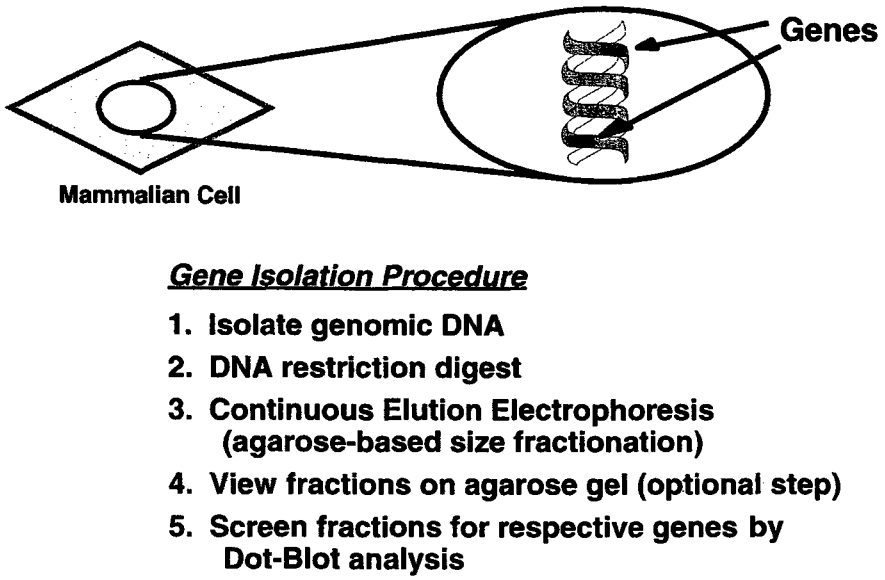

FIGURE 4 Schematic representation of the steps in target gene enrichment by CEE. Reproduced with permission.

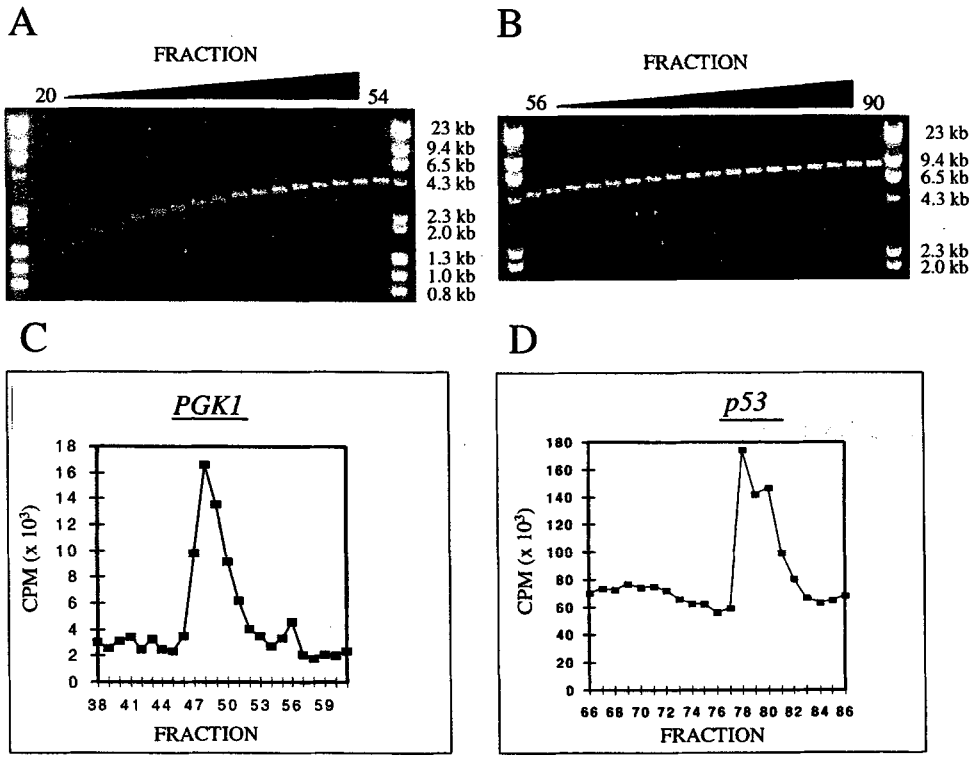

FIGURE 5 Determination of CEE fractions containing target genes. 300 μg aliquots of *Bam*H I-digested total human genomic DNA were fractionated by CEE. 90 ng aliquots of every other CEE fraction were analyzed by 0.7% agarose gel electrophoresis (Gel [A], fractions 20–54; gel [B], fractions 56–90) to assess the DNA fragment size range per fragment. Thirty ng aliquots were dot-blotted to nylon membranes, then hybridized with α-[^{32}P]-labeled probes ([C]: *PGK1* probe; [D]: *p53* probe). Hybridization signal intensities were quantified by PhosphorImager analysis. Reproduced with permission.

the *p53* (exons 3–11) and *PGK1* genes were determined by dot-blot analysis (Figure 5C and D) then used as LMPCR substrates. Use of the target gene-enriched DNA as LMPCR substrate resulted in an average 24-fold enhancement of LMPCR-derived base damage signal intensity as compared to non-enriched total genomic DNA (Figure 6).

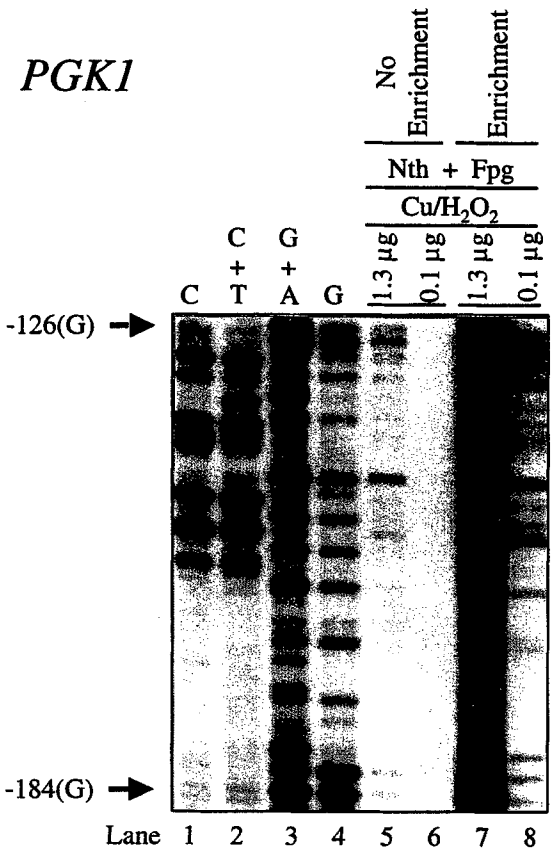

FIGURE 6 Autoradiogram of DNA fragments produced by LMPCR analysis (primer set G, transcribed strand) of damage induced in the promoter region of human *PGK1* by treating purified genomic DNA with 50 µM Cu(II)/100 µM ascorbate/0.5 mM H_2O_2 in phosphate buffer for 30 min at 37°C. Lanes 5, 6, non-enriched treated DNA, digested with Nth and Fpg proteins after treatment; lanes 7, 8, treated DNA, digested with Nth and Fpg proteins after treatment, then enriched by CEE fractionation; lanes 1–4, non-enriched, non-treated DNA subjected to standard Maxam–Gilbert cleavage reactions. Reproduced with permission.

DISCUSSION

We have observed the frequency patterns of DNA base damage in two genes on separate chromosomes induced *in vivo* by H_2O_2 and *in vitro* by H_2O_2 in the presence of copper, iron, or chromate ions plus ascorbate. The data indicate two generalizations: (1) DNA base damage caused by any one of the three metal ions, Cu(II), Fe(III), or Cr(VI), *in vitro* is remarkably similar to each other, as well to the *in vivo* base damage induced by H_2O_2, and (2) DNA base damage frequency *in vivo* is enhanced by metals or metal-like ligands which reside at extranuclear sites, but are mobilized by severe oxidative stress, and migrate to the nucleus.

DNA-reactive species generated from H_2O_2 in association with soluble[10] or loosely-bound redox cycling ligands[15] predominately cause frank strand breaks, whereas cycling ligands bound intrahelically to DNA bases predominately cause modified bases. *In vivo* H_2O_2 treatment predominantly causes base damage, suggesting that damage induced in cultured human fibroblasts by H_2O_2 involves redox cycling ligands bound to DNA bases in the helix. The near identity of the *in vivo* and *in vitro* transition metal ion-catalyzed damage patterns suggest that these DNA-bound redox cycling ligands are transition metal ions. Additionally, the damage frequency patterns indicates that the principal determinant of the probability of a H_2O_2-associated damaging event occurring at any position is the primary DNA sequence. Chromatin structure, with the exception of the transcription factor footprints,[16] is only a minor determinant of base damage probability.

The nucleotide-resolution maps of DNA base damage induced *in vitro* in the presence of Cu(II), Fe(III), or Cr(VI) transition metal ions in two genes, were remarkably similar. This similarity suggests a model in which the local binding site occupancy rate and the local geometry of the metal ion–DNA–peroxo coordination complex

determine the probability of a damage event at each position.

Experiments with isolated fibroblast nuclei indicated that nuclei have insufficient bound transition metal to induce base damage detectable by neutral denaturing gel electrophoresis; exogenous transition metal ions must be added to the nuclei to produce measurable base damage. Sufficient DNA-bound redox cycling ligands are likely to be present *in vivo*, but are normally sequestered at extranuclear sites. The extreme oxidative stress caused by exposure of cells to 50 mM of H_2O_2 may have liberated normally sequestered extranuclear transition metal ions into the nucleus, allowing chromatin to show a transition metal ion-saturated pattern. Oxidant-mediated liberation of cytoplasmic sequestered transition metal ions has been observed in other model systems.[17] Larger non-sequestered transition metal ion pools caused by H_2O_2 exposure would most likely facilitate DNA binding at low affinity binding sites to a greater extent than high affinity binding sites, which may become saturated with metal ions; therefore, this effect may tend to "smooth out" the differences in DNA damage frequency between certain nucleotide positions.

Analysis of H_2O_2-mediated *in vivo* DNA damage frequency patterns in human fibroblasts at nucleotide resolution indicate that the principle determinant of the local DNA damage probability is the primary DNA sequence. Chromatin structure has a very minor effect. Furthermore, damage caused by H_2O_2 *in vivo* is mediated by DNA-bound transition metal ions (or other redox cycling ligands with similar characteristics), that in addition to residing within a cell's nucleus, also reside at extranuclear sites. The damage frequency patterns do not permit definitive determination of which transition metal ions are involved in *in vivo* DNA damage production because the damage frequency pattern is independent of the type of transition metal ion, at least with respect to copper, iron, and chromium ions. Recent improvements, such as the enhancement of LMPCR-derived damage signal intensity by target gene enrichment, is permitting the mapping of base damage induced by non-cytotoxic exposures of fibroblasts to H_2O_2 *in vivo*; such studies are currently ongoing in our laboratory. Also, due to the enhancement of damage signal intensity, non-phenol extractions are conducted providing better signal-to-noise ratios. Our data simplifies the field of oxidative base damage by showing that the pattern of base damage is independent of the redox cycling metal (or metal-like ligand).

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